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Proteolytic Activity of the Ruminal Bacterium *Butyrivibrio fibrisolvens*

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The proteolytic activity of *Butyrivibrio fibrisolvens*, a ubiquitously distributed bacterial species in the gastrointestinal tracts of ruminants and other mammals, was characterized. The relative proteolytic activity (micrograms of azocasein degraded per hour per milligram of protein) varied greatly with the strain: 0 to 1 for strains D1, D16f, E21C, and X6C61; 7 to 15 for strains IL631, NOR37, S2, LM8/1B, and X10C34; and 90 to 590 for strains 12, 49 H17C, CF4c, CF3, CF1B, and R28. The activity levels of the last group of strains were equal to or greater than those found with *Bacteroides amylophilus* or *Bacteroides ruminicola*. With the exception of strain R28 activity, 90% or more of the proteolytic activity was associated with the culture fluid and not the cells. Strain 49 produced proteolytic activity constitutively, but the level of activity (units per milligram of protein) was modulated by growth parameters. With various carbohydrates added to the growth medium, the proteolytic activitites of strain 49 were positively correlated with the growth rate. However, when the growth rate varied with the use of different nitrogen sources, a similar correlation was not found. The highest activity level was observed with Casamino Acids (1 g/liter), but this level was reduced by ca. 70% with Trypticase (BBL Microbiology Systems, Cockeysville, Md.) or casein (1 g/liter) and by 85% with ammonium chloride (10 mM) as the sole nitrogen source. The addition of ammonium chloride (1 to 10 mM) to media with low levels of Casamino Acids or Trypticase resulted in lower proteolytic activities but not as low as seen when the complex nitrogen sources were increased to high levels (20 g/liter). Proteolytic activity was affected slightly if at all by freezing and increased proportionally with the assay temperature up to 47°C. No precise optimal pH was observed, and the highest activities were in the pH range of 5.5 to 7.0. The proteolytic activity was insensitive to oxygen, and either dithiothreitol or L-cysteine inhibited activity up to 40%. The effects of protease inhibitors indicated the proteolytic activities of the culture fluid and cells, which are the same and are of a serine protease type. Preliminary data from initial purification procedures suggest that the proteolytic activity in the culture fluid consists of a low-molecular-weight protein that is associated with carbohydrate material.

In the rumen, proteins from ingested feedstuffs are extensively degraded (50 to 70%) along with plant cell polysaccharides. The major protein source for the animal comprises the microbial cells formed in the rumen, with small amounts coming from undegraded feed proteins or their partial hydrolysis products. Ruminal protein degradation is a composite of several microbial processes, including protein hydrolysis, peptide degradation, amino acid deamination, and the fermentation of amino acid carbon skeletons. Much of the information on these microbial activities has been obtained from measurements of ruminal contents directly or of mixed bacterial populations obtained from the contents. Since these data represent the combined effects of many enzymes produced by a variety of microorganisms, available information is biochemically imprecise and difficult to interpret. However, such studies have shown that ruminal contents have several types of proteases present and that most proteolytic activity is associated with the bacteria (13).

About 12 to 38% of the total bacterial population are strains having proteolytic activities (14, 20). The predominant proteolytic members belong to the major genera of saccharolytic ruminal bacteria. Proteolytic species that use proteins as a sole source of carbon and energy are usually facultative anaerobes which are numerically insignificant in the rumen (2, 8, 29). The important proteolytic species are *Bacteroides amylophilus*, *Bacteroides ruminicola*, and *Butyrivibrio fibrisolvens* (9, 16, 17, 20, 24, 25). The proteolytic activity of *Bacteroides amylophilus* has been studied in some detail (5, 6, 10, 26, 33). The activity is cell associated but is released by lysis of cells during the stationary-growth phase and may consist of one or two trypsinlike proteases. *Bacteroides ruminicola* also has cell-associated proteolytic activity that is released upon cell lysis. The activity is oxygen sensitive and may consist of a mixture of serine, thiol, and carboxyl proteases (23). In contrast to the previous two organisms, virtually nothing is known about the proteolytic activity of *B. fibrisolvens*, even though it is often the most numerous proteolytic organism under many dietary conditions (9, 20, 25). In this paper, we show the distribution of proteolytic activity among *B. fibrisolvens* strains, describe the general properties of the proteolytic activity of strain 49, and present the effects of various growth parameters on the production of this activity.

MATERIALS AND METHODS

Organisms. *B. fibrisolvens* 49, A33, D1, and C14; *Bacteroides ruminicola* B_14, and 23; *Eubacterium ruminantium* GA195; *Bacteroides amylophilus* H18; *Anaerovibrio lipolytica* 7553; *Lachnospira multiparus* 40; *Selenomonas ruminantium* HD4; and *Streptococcus bovis* JB1 were obtained from the culture collection of M. P. Bryant, Department of Dairy Science, University of Illinois, Urbana. *B. fibrisolvens* H17c, E21c, CF4c, CF3, CF1B, and D16f were provided by B. A. Dehority, Agricultural Research and

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TABLE 1. Composition of RF, RS, and DF growth media[a]

Ingredient	Medium (amt/100 ml)		
	RF	RS	DF
Carbohydrate solution (20% [wt/vol])[b,c]	1.0 ml	1.0 ml	1.0 ml
Trypticase	0.1 g	0.1 g	—[d]
Yeast extract	—	0.2 g	—
Clarified rumen fluid[e]	20.0 ml	—	—
Mineral 1[f]	4.0 ml	4.0 ml	4.0 ml
Mineral 2[f]	4.0 ml	4.0 ml	—
Mineral 3[g]	—	—	4.0 ml
Volatile fatty acid solution[f]	0.2 ml	0.3 ml	0.3 ml
L-Cysteine–HCl solution (5.0% [wt/vol])[c]	1.0 ml	1.0 ml	—
$Na_2S \cdot 9H_2O$ solution (5.0% [wt/vol])[c]	—	—	1.0 ml
Na_2CO_3 solution (8.0% [wt/vol])[c]	5.0 ml	5.0 ml	5.0 ml
Resazurin solution (0.1% [wt/vol])	0.1 ml	0.1 ml	0.1 ml
Hemin-naphthoquinone solution[h]	1.0 ml	1.0 ml	1.0 ml
R-1 salts solution[i]	—	0.1 ml	0.1 ml
Vitamin solution[c,j]	—	—	1.0 ml

[a] Prepared under CO_2; pH adjusted to 6.8 with KOH before autoclaving.
[b] Carbohydrate was glucose unless indicated otherwise.
[c] Prepared under N_2 (CO_2 for $NaCO_3$ solution) and added as a separate, sterile solution to cooled medium.
[d] —, Not added.
[e] Prepared as fraction 2 as described by Bryant and Robinson (15).
[f] Prepared as described by Caldwell and Bryant (18).
[g] Same as mineral 2 but with 5.58 g of Na_2SO_4 in place of $(NH_4)SO_4$.
[h] Prepared as described by Gomez-Alarcon et al. (22).
[i] Prepared as described by Hespell and Canale-Parola (27).
[j] Contained per 100 ml of 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesultonic acid) buffer (pH 7.5): biotin, folic acid, *p*-aminobenzoic acid, and cyanocobalamin (2.5 mg each) and calcium pantothenate, nicotinamide, riboflavin, thiamine hydrochloride, and pyridoxamine (20 mg each). The solution was filter sterilized (pore size, 0.22 μm) and stored in a dark container.

Development Center, The Ohio State University, Wooster. *B. fibrisolvens* ACTF2, 12, X10C34, and X6C61 were obtained from N. O. VanGylswyk, Council for Scientific and Industrial Research, National Chemical Research Laboratory, Pretoria, Union of South Africa. *B. fibrisolvens* B835, IL631, S2, LM8/1B, and NOR 37 were supplied by G. P. Hazlewood, Department of Biochemistry, Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge, England. *B. fibrisolvens* R28 and *Bdellovibrio stolpii* UKi2 were obtained from the culture collection of R. B. Hespell.

Media and growth conditions. For most experiments, *B. fibrisolvens* 49 and other ruminal bacteria were grown in the complex media (rich [RS] and rumen fluid [RF]) shown in Table 1. For studies on the effects of nitrogen sources on the proteolytic activity of strain 49, a chemically defined medium (DF) was used (Table 1). Initial attempts to cultivate strain 49 in similar media containing pyridoxine as a component of the B-vitamin mixture resulted in poor growth. When pyridoxine was replaced by pyridoxamine, DF medium containing ammonium chloride as the sole source of nitrogen supported growth comparable to that observed in RS or RF media.

All cultures were grown anaerobically in batch cultures at 39°C. Growth was monitored spectrophotometrically (Spectronic 70; Bausch & Lomb, Inc., Rochester, N.Y.) by determining the optical densities of cultures at 660 nm.

Gelatin liquefaction. Gelatin liquefaction was determined as described by Holdeman et al. (28). Ruminal bacteria were grown overnight in RF medium supplemented to contain (wt/vol) 12% gelatin, 0.0001% hemin, 0.2% maltose, 0.2% cellobiose, 0.1% glycerol, and 0.1% fructose. Cultures where the medium failed to solidify when refrigerated were scored as positive for gelatin liquefaction.

Casein degradation. The ability of ruminal bacteria to form clearing zones in casein-containing agar medium was used as a qualitative test for casein degradation. The growth medium used was essentially the same as for gelatin liquefaction, except that gelatin was deleted and 1.5% (wt/vol) agar was added. The medium was prepared and dispensed in petri dishes in the anaerobic glove box by the techniques described by Leedle and Hespell (32). The solidified medium was then overlaid with a casein-agar preparation: 5% (wt/vol) casein, 4% (vol/vol) mineral 1 (Table 1), 4% (vol/vol) mineral 2, and 1% (wt/vol) agar. Bacteria were spot-inoculated into the casein overlay with sterile Pasteur pipettes. Plates were transferred to anaerobic incubation vessels, placed under a 100% carbon dioxide atmosphere, and incubated at 39°C until visible colony formation occurred (generally about 3 days). Casein degradation was determined by flooding the plate with hydrochloric acid (1 N), which precipitated undegraded casein. Colonies that showed zones of clearing were scored as positive for casein degradation.

Culture harvesting. Logarithmic-phase cultures were used for most experiments. Cultures were harvested by centrifugation (6,000 × *g*) for 30 min at 4°C, and cell-free culture fluids were retained for analyses. Cell pellets were resuspended in 1/10 of the original culture volume of either 100 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] buffer (pH 6.8) or 100 mM potassium phosphate buffer (pH 6.8) and were retained for analyses. These manipulations were performed without precautions for maintaining anaerobiosis, except when an assay of proteolytic activity under anaerobic conditions was required. In the latter case, cultures to be harvested were transferred to an anaerobic glove box where samples were removed and transferred to centrifuge bottles with sealing caps Sorvall Products, Du Pont Co., Wilmington, Del.). The bottles were removed from the glove box for centrifugation and then returned to the glove box; the supernatant fluids were decanted, and the cell pellets were resuspended in the appropriate anaerobic buffer. Analysis of these samples for proteolytic activity was conducted immediately. All glassware, plastic ware, and utensils required for these manipulations were placed in the glove box at least 24 h before their use to ensure the removal of oxygen from these materials.

Experiments on the production of the proteolytic activity of *B. fibrisolvens* 49 and on related nutritional influences required sample collection over periods of several hours or even days. Cell-free culture fluid and resuspended cell samples were stored frozen at −30°C under an air atmosphere. The freezing of samples for 2 weeks resulted in no detectable loss of proteolytic enzyme activity, and storage for 5 months caused only about 20% loss of enzyme activity. All enzyme assays were performed within 2 weeks of the time of sample collection. Frozen enzyme samples were identical to unfrozen samples with regard to the pH optimum and sensitivity to proteolytic enzyme inhibitors.

Protease assay. Protease activity of samples was determined spectrophotometrically with azocasein as the substrate (13). The assay mixtures contained 0.5 ml of substrate (0.8% [wt/vol] azocasein in 100 mM potassium phosphate or PIPES buffer [pH 6.8]) and 0.5 ml of the enzyme source in

1.8-ml Eppendorf-type microcentrifuge tubes. Assay mixtures were incubated for 3 h at 39°C. The reaction was stopped by the addition of 0.5 ml of cold 1.5 M $HClO_4$ to each tube, which then was held on ice for 30 min. The precipitated protein was removed by centrifugation (12,000 × *g*) for 5 min, and 1 ml of supernatant fluid was withdrawn and combined with an equal volume of 1 N NaOH. The concentration of acid-soluble azopeptides in the resultant solution was determined by optical density measurement at 440 nm, with 1 optical density unit equal to 320 μg/ml. Control assays were performed by incubating enzyme samples and the azocasein substrate separately and by combining these solutions at the time of acid addition. One proteolytic enzyme unit equaled 1 μg of azocasein digested per h under the above-specified conditions. Because growth varied with growth conditions and culture fluids contained low protein concentrations (1 to 5 μg/ml), proteolytic activities were reported as units per milligram of cell protein (relative activity) to accommodate these variations. All enzyme assays were performed at least in duplicate. Validity of the activity assay was tested for linearity with respect to time and enzyme concentration. All assays were conducted for the linear portion of these relationships. The addition of 20 g of Casamino Acids (Difco Laboratories, Detroit, Mich.) or Trypticase (BBL Microbiology Systems, Cockeysville, Md.) per liter did not cause interference in the assay.

For the assay of proteolytic enzyme activity under anaerobic conditions, the azocasein substrate was dissolved in anaerobic buffer (100 mM PIPES or potassium phosphate [pH 6.8] boiled under nitrogen). Enzyme samples and the substrate solution were transferred to the anaerobic glove box (95% argon, 5% hydrogen atmosphere), and 1.0 ml of the enzyme sample was combined with 1.0 ml of the azocasein substrate in test tubes (13 by 100 mm). Test tubes were closed with no. 00 black rubber stoppers, removed from the glove box, and incubated at 39°C. After 3 h, the tubes were opened and 1.0 ml of the assay mixture was transferred to 1.8-ml microcentrifuge tubes. All subsequent manipulations were as described above.

Attempts were made to measure proteolytic activities by the hydrolysis of synthetic substrates. Hydrolysis of *N*-α-benzoyl-*l*-arginine-*p*-nitroanilide (410 nm), *N*-α-benzoyl-DL-arginine-β-naphthylamide (335 nm), *N*-α-carbobenzoxy-*l*-lysine-*p*-nitrophenyl ester (410 nm), *N*-benzoyl-*p*-tyrosine-*p*-nitroanilide (410 nm), *N*-acetyl-DL-phenylalanine-β-naphthyl ester (328 nm), or *N*-carbobenzoxy-*l*-phenylalanine-*p*-nitrophenyl ester (410 nm) was measured spectrophotometrically (37). The assay mixtures contained 0.2 ml of substrate (25 mM in dioxane) and 2.0 ml of cell-free culture fluid. Substrate and enzyme samples were mixed in cuvettes, and the change in absorbance was monitored for 30 min. The addition of dioxane to the cell-free culture fluid did not inhibit proteolytic activity as measured by azocasein digestion.

Proteolytic enzyme inhibitors. Proteolytic enzyme inhibitors were dissolved in either 100 mM potassium phosphate buffer (pH 6.8) or ethanol (100%), and 0.1 ml of inhibitor solution was added to the assay mixtures to yield the final inhibitor concentrations (see Table 6). Phenylmethylsulfonyl fluoride (PMSF), tosylamide phenylethyl chloromethyl ketone (TPCK), tosyl-*l*-lysine chloromethyl ketone (TLCK), and *o*-phenanthroline were each dissolved in ethanol. All other inhibitors were dissolved in phosphate buffer. Inhibitors were added to the enzyme samples 5 min before the addition of the azocasein substrate. The inhibition of protease activity was determined by comparing the activity of assays containing inhibitors with the activity of controls containing 0.1 ml of buffer or ethanol. The effects of the inhibitors on the activities of trypsin, chymotrypsin, papain, or pepsin were determined under similar assay conditions to measure the effectiveness of the protease inhibitors. The effectiveness of metal chelators as inhibitors of metalloprotease activity was determined by measuring the effect of these compounds on the extracellular proteolytic activity of a metalloprotease-producing organism, *Bdellovibrio stolpii* UKi2. Cultures of *Bdellovibrio stolpii* were grown and sampled as described by Gloor et al. (21). In all cases, the inhibitors effectively blocked the activity of the appropriate proteolytic enzyme.

DEAE-Sephadex treatment of cell-free culture fluid. Anion-exchange chromatography was used to concentrate and partially purify proteolytic activity from the *B. fibrisolvens* 49 culture fluid. Settled DEAE-Sephadex A-25 slurry (50 ml) that had been equilibrated with starting buffer (100 mM PIPES; pH 6.8) was combined with 1 liter of cell-free culture fluid. After being stirred for 1 h at 4°C, solids were allowed to settle and the supernatant fluid was removed. Approximately 85% of the proteolytic activity was removed from the solution in this manner. Additional buffer was added, and the slurry was poured into a chromatographic column (1 by 100 cm). The column was washed with buffer until no further 280-nm-absorbing material was eluted. A linear NH_4Cl gradient from 0 to 2 M in 100 mM PIPES (pH 6.8) was applied, and proteolytic activity was eluted. All manipulations were performed in a cold room at 4°C.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as follows. Cell-free culture fluids were concentrated by ultracentrifugation (165,000 × *g*) for 2 h at 4°C and prepared for electrophoresis by suspending pellets in Tris-glycine buffer (25 mM Tris, 200 mM glycine; pH 8.5) containing 1% sodium dodecyl sulfate and 10 mM dithiothreitol. Samples were denatured by heating at 100°C for 10 min, followed by alkylation with iodoacetamide (10 mM) at 50°C for 5 min to prevent the reoxidation of sulfhydryls. Vertical slab gels (12 cm by 14 cm by 1.5 mm) were prepared to contain 10% (wt/vol) acrylamide by the method of Laemmli (31). Samples (20 μl) were loaded into the wells and electrophoresed for 18 h (40 V, 6 mA). The gels were fixed in 1.0 M $HClO_4$ for 20 min and stained by the Gelcode silver stain method (Pierce Chemical Co., Rockford, Ill). The estimation of molecular weights of unknown proteins was performed by comparing them with the gel migration distances of the following known proteins: insulin (3 kilodaltons [kDa]), bovine trypsin inhibitor (6.2 kDa), cytochrome *c* (12.3 kDa), lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), α-chymotrypsinogen (25.7 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase *b* (97.4 kDa), and myosin (200 kDa).

Determination of protein and carbohydrate concentrations. Cell protein concentrations were determined by the method of Lowry et al. (34) after the hydrolysis of cells in base (0.1 N NaOH) at 70°C for 30 min. The protein content of cell-free materials was estimated with the Coomassie blue dye-binding method of Bradford (12). Cytochrome *c* (horse heart) was used as the standard for protein determinations. Carbohydrate concentrations were determined by the phenol-sulfuric acid method (3). Glucose was used as the standard.

Chemicals. Unless indicated otherwise, all chemicals, buffers, and reagents were of analytical reagent or biological grade and were purchased from Sigma Chemical Co., St. Louis, Mo. Bacteriological media and constituents were purchased from either Difco Laboratories or BBL Microbi-

TABLE 2. Proteolytic activity levels and distribution between cells and culture fluid in *Bacteroides amylophilus*, *Bacteroides ruminicola*, and *B. fibrisolvens*[a]

Species and strain	Total activity (U/ml of culture)	Relative activity (U/mg of cell protein)	% Distribution in:	
			Fluid	Cells
Bacteroides amylophilus H18	47	400	8	92
Bacteroides ruminicola B_14	21	100	28	72
Butyrivibrio fibrisolvens				
CF4c	54	336	92	7
CF3	33	231	91	9
CF1B	42	186	95	5
49	26	125	87	13
49[b]	72	570	95	5
12	40	250	88	12
H17c[b]	64	590	91	9
AcTF2	12	90	85	15
R28	28	172	59	41
LM8/1B	15	95	77	23
IL631	7	29	79	21
NOR37	7	32	84	16
S2	11	47	91	8
C14	9	54	75	25
X10C34	8	ND[c]	80	20
X6C61	0	ND	ND	ND
E21C	1	6	ND	ND
D16f	1	5	ND	ND
D1	1	10	100	ND

[a] All strains were grown on RS medium with 0.3 g of Trypticase per 100 ml of medium. Cells were harvested at the late-logarithmic-growth phase. All values are averages of duplicate samples from two replicate experiments.
[b] Grown on RF medium.
[c] ND, Not determined.

ology Systems. DEAE-Sephadex was obtained from Pharmacia, Uppsala, Sweden. Molecular weight standards were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

RESULTS

Proteolytic activity levels of ruminal species. A wide variety of ruminal bacterial species were screened for proteolytic activity by liquefaction of gelatin broths and by production of clearing zones around colonies on plates of RF medium containing casein. It was observed that many strains were consistently negative for both tests. Such strains included *Streptococcus bovis* JB1, *Selenomonas ruminantium* HD4, *E. ruminantium* GA195, *A. lipolytica* 7553, and *L. multiparous* 40. In contrast, known proteolytic strains, such as *Bacteroides ruminicola* 23 and B_14 or *Bacteroides amylophilus* H18, gave clear positive results with both tests, as did *B. fibrisolvens* 49 and A38. The zones of casein hydrolysis, however, varied considerably with species. *Bacteroides amylophilus* strains produced large zones around each colony, whereas strains of both *Bacteroides ruminicola* and *B. fibrisolvens* showed only small but distinct zones around the colony border.

The proteolytic activity levels of *B. fibrisolvens* strains were quantitatively determined and compared with those of the *Bacteroides* species (Table 2). *Bacteroides amylophilus* H18 had a rather high proteolytic activity level, and as expected (5), it was almost all cell associated. However, with *Bacteroides ruminicola*, only about 70% of the activity was cell associated. *B. fibrisolvens* CF4c, CF3, CF1B, 49, 12, and R28 all produced total proteolytic activity levels equal to or higher than those of the *Bacteroides* species. A number of other *Butyrivibrio* strains showed low levels of proteolytic activity. As shown by the data for strain 49, the growth medium can influence the relative activities. Except for the activity of strain R28, most of the proteolytic activity of *B. fibrisolvens* strains was in the culture fluid and was not cell associated, as was found with *Bacteroides* species. The proteolytic activity levels of strain 49 were monitored throughout the culture growth cycle (Table 3). The data showed that regardless of the stage of growth, 95% or more of the proteolytic activity was always found in the culture fluid and the accumulation of activity generally paralleled growth.

Because of its relatively high proteolytic activity level and good growth properties, *B. fibrisolvens* 49 was chosen for more detailed studies of its proteolytic activity.

Effect of carbohydrate source on proteolytic activity. As with most *Butyrivibrio* strains, strain 49 can grow on a wide range of carbohydrates that serve as energy and carbon sources. The growth rates of the cultures varied considerably from 0.22 to 0.74 per h, depending on the particular carbohydrate provided for growth (Table 4). Similarly, the total proteolytic activity levels of these cultures were measurably different, with a tendency for rapidly growing cells to exhibit higher activity levels. An analysis of the data indicated a positive correlation ($r = 0.75$; $P < 0.01$) between the growth rate and the total culture proteolytic activity.

Effect of nitrogen source on proteolytic activity. *B. fibrisolvens* 49 grew well on a defined medium (DF) (Table 1), but both the maximal growth levels and specific growth rates varied with the particular nitrogen source added to the growth medium (Table 5). The highest growth levels were

TABLE 3. Distribution of proteolytic activity between cells and culture fluid during growth of *B. fibrisolvens* 49

Optical density at 660 nm[a]	Proteolytic activity (U/ml)	% of total culture activity
0.067		
Cells	0.40	3
Fluid	13.32	97
0.174		
Cells	1.76	4
Fluid	42.24	96
0.300		
Cells	2.39	4
Fluid	64.65	96
0.417		
Cells	3.28	4
Fluid	76.35	96
0.517		
Cells	3.76	4
Fluid	84.16	96
0.640		
Cells	5.27	5
Fluid	109.51	95
0.640		
Cells	6.11	5
Fluid	106.90	95

[a] Of culture (RF medium) at time of harvest.

TABLE 4. Effect of added carbohydrate source on the proteolytic activity of *B. fibrisolvens* 49[a]

Carbohydrate	Specific growth rate per h[b]	Optical density at 660 nm[c]	Mean relative activity[d] ± SD (U/mg of cell protein)
Glucose	0.69	0.560–0.630	355 ± 76
Cellobiose	0.25	0.570–0.620	287 ± 8
Maltose	0.30	0.650–0.725	277 ± 21
Fructose	0.62	0.490–0.500	423 ± 18
Sucrose	0.74	0.540–0.690	311 ± 12
Xylose	0.22	0.560–0.680	218 ± 1

[a] Cultures were grown on RS medium with the indicated carbohydrate source and were harvested just when growth ceased.
[b] Measured during logarithmic growth phase.
[c] At time of harvest.
[d] Activity in the cell-free fluid. Values are from duplicate samples from two replicate experiments.

observed with Trypticase, followed by casein and then ammonium chloride. Although Casamino Acids supported good growth rates, the overall growth yields were less than half of those found with the other nitrogen sources. There was no correlation between growth rates and proteolytic activity levels. The highest proteolytic activity level was found in cultures grown with low levels (1 g/liter) of Casamino Acids, but only about 5% of this level of activity was found with cultures containing high levels (20 g/liter) of Casamino Acids. Similar results were found with Trypticase, but the change was less. Low levels of Trypticase resulted in cultures having proteolytic activity levels which were about equal to that found with casein but which amounted to only 35% of those found with low levels of Casamino Acids.

B. fibrisolvens produces ammonia from complex nitrogen sources (11). With ruminal bacteria, ammonia can drastically affect levels of intracellular nitrogen metabolism enzymes, such as glutamate dehydrogenase, glutamine synthetase, or urease (38, 39, 40). The addition of ammonium chloride (1 to 10 mM) to cultures with casamino acid-based DF medium resulted in about a 70% lowering of relative proteolytic activity levels. These levels were comparable to those observed for low levels of Trypticase (Table 5). In contrast, the inclusion of ammonium chloride in cultures with Trypticase-based DF medium resulted in little reduction (less than 15%) in proteolytic levels. Regardless of the levels of casamino acids or Trypticase present, the inclusion of ammonium chloride in the medium had little (10% or less) or no effect on the specific growth rates of the cultures.

Chemical properties of proteolytic activity. A variety of synthetic substrates has been used to measure the activity of proteases. However, the proteolytic activity of *B. fibrisolvens* could be measured with sufficient sensitivity and reproducibility only when azocasein was used as the synthetic substrate. Relative to azocasein hydrolysis, little or no activity could be detected with trypsinlike or chymotrypsinlike substrates or *p*-nitroanilide esters of several different amino acids.

Although many enzymes from anaerobic bacteria are often inhibited by oxygen, the proteolytic activity in culture fluids of *B. fibrisolvens* was relatively stable. When procedures were used to exclude oxygen from all assay conditions and during the collection of the culture fluid or cells, activity levels were not measurably different (less than 5%) from those obtained under aerobic procedures. In fact, the inclusion of dithiothreitol (1.5 mM) in the assay mixture inhibited proteolytic activity by 25 to 35% regardless of whether aerobic or anaerobic procedures were used. Dithiothreitol is a metal-chelating agent and can be inhibitory to metalloproteases (4). Inclusion of EDTA (5 mM) in the assay mixture had little effect on enzyme activity, and inclusion (1.25 mM) of the chloride salts of calcium, manganese, or magnesium stimulated activity by only 10% or less. On the other hand, chloride salts of zinc, nickel, or cobalt resulted in about a 50% loss of activity. These losses could be partially overcome if dithiothreitol was also included in the assay mixture.

Both the assay temperature and pH influenced the proteolytic activity of the culture fluid. Increasing the assay temperature from 25 to 47°C resulted in a linear, 4.5-fold increase of activity. At assay temperatures above 47°C, an almost linear loss of activity with temperature increases was observed, with less than 10% of the maximal activity (47°C) measurable at 70°C. The pH of the assay mixture was varied from 4 to 11, with different buffers having distinct pK_a values. No precise optimal pH was observed for the culture fluid proteolytic activity, and the highest activity levels were found from pH 5.5 to 7.0 (Fig. 1). A similar pH profile was found for the cell-associated proteolytic activity.

Effect of inhibitors on proteolytic activity. A diverse range of compounds known to inhibit various proteases were tested for their effects on the proteolytic activity of *B. fibrisolvens* 49 (Table 6). Metalloprotease inhibitors (EDTA and *o*-phenanthroline) and pepstatin, an inhibitor of carboxyl proteases, had no effect on the proteolytic activity of *B. fibrisolvens*. Thiol proteases are inhibited by iodoacetamide or *p*-chloromercuribenzoate, but only the latter compound was found to be inhibitory at high concentrations. Sodium sulfide, L-cysteine, or dithiothreitol are often stimulatory to thiol proteases; however, the proteolytic activity of neither the culture fluid nor the cells was found to be stimulated by low concentrations of these reducing agents. TPCK and TLCK inhibit serine proteases with trypsin- or chymotrypsinlike specificities (42). Neither compound inhibited the cell-associated proteolytic activity, and they were only slightly, if at all, inhibitory to the culture fluid activity. In addition, trypsin inhibitor I-S was also found to be ineffective. PMSF causes a strong inhibition of serine proteases by reacting with the hydroxyl group of a serine residue in the active site (4, 42). Both the culture fluid and cell-associated proteolytic activities were almost completely eliminated when PMSF was included in the assay mixture.

Partial purification of proteolytic activity. Routinely, the culture fluid contained only 1 to 7 μg of total protein per ml. Attempts to precipitate the proteolytic activity consistently by ammonium sulfate treatment were not successful, and

TABLE 5. Effect of added nitrogen source on the proteolytic activity of *B. fibrisolvens* 49[a]

Nitrogen source	Concn	Specific growth rate per h	Mean relative activity[b] ± SD (U/mg of cell protein)
Ammonium chloride	10 mM	0.483	122 ± 9
Casamino Acids	1 g/liter	s0.414	934 ± 41
Casamino Acids	20 g/liter	0.410	56 ± 7
Trypticase	1 g/liter	0.557	286 ± 20
Trypticase	20 g/liter	0.847	37 ± 14
Casein	1 g/liter	0.240	268 ± 28

[a] Cultures were grown on DF medium with indicated nitrogen source and were harvested at late-logarithmic growth phase.
[b] Values are from duplicate samples from three replicate experiments.

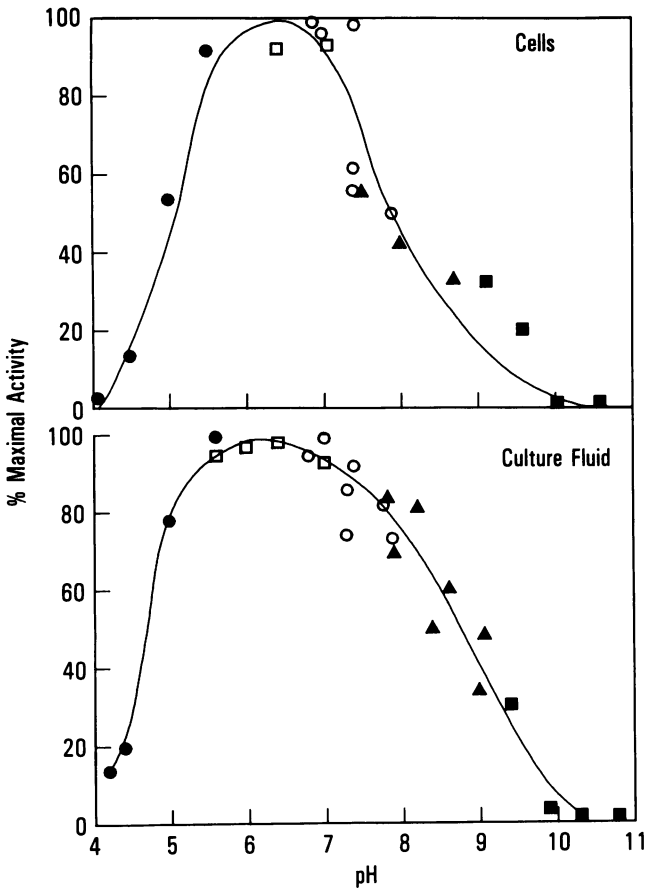

FIG. 1. Effect of pH on the proteolytic activity of *B. fibrisolvens* 49. Cultures were grown on RF medium (Table 1), and the cells were harvested by centrifugation (6,000 × *g*) for 30 min. The cells were resuspended to 1/10 of the original volume in appropriate buffer for desired pH. All buffers were 50 mM: acetate (●), citrate-phosphate (□), phosphate (○), Tris (▲), and CO_2-carbonate (■). The data are averages from two separate replicate experiments.

less than 50% of the original activity could be recovered from the pellet obtained at 60% saturation. However, passage of the culture fluid through a column of DEAE-Sephadex resin resulted in the binding of ca. 85% of the proteolytic activity. With an ammonium chloride gradient, most of the recovered activity was eluted at ca. 0.3 M, but the elution times were rather long, and less than 5% of the original culture activity could be recovered. Batch treatment of the culture fluid with DEAE-Sephadex resin, followed by batch elution, increased recoveries to about 20% of the original activity, but the purification levels were less than twofold.

Chemical analysis of the eluted material revealed not only the presence of protein but also substantial amounts of carbohydrate. The protein-carbohydrate material behaved as if it had a high molecular weight, because it was retained by 100-kDa molecular filters. Culture fluid samples were subjected directly to centrifugation. With low-speed centrifugation (20,000 × *g*) for 30 min, only ca. 10–15% of the total proteolytic activity could be recovered from the opaque pellet, but some 80% of the activity could be recovered from the translucent pellet obtained from high-speed centrifugation (165,000 × *g*) for 2.5 h. With the pellet from ultracentrifugation, chemical analysis indicated the presence of carbohydrate plus protein and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed the presence of one densely staining protein band of 30 kDa and several faint bands around 75 to 120 kDa. Preliminary sugar analysis of the carbohydrate indicated the presence of two major sugars (glucose and galactose) and several minor ones.

DISCUSSION

B. fibrisolvens can be considered a proteolytic species because a majority of the strains tested were proteolytic (Table 2). Both by total and relative activities produced, several strains of *B. fibrisolvens* were as proteolytic as *Bacteroides amylophilus* or *Bacteroides ruminicola* were. In contrast to the activity of these latter two organisms, the proteolytic activity of *B. fibrisolvens* was extracellular (Table 2), regardless of growth stage (Table 3). By comparison, however, about 75% of the proteolytic activity found with rumen contents can be associated with the particulate fraction and not with the fluid (13). In the rumen, the majority of *B. fibrisolvens* cells are probably attached to feed particles; in vitro studies have indicated that this organism rapidly attaches to cellulose particles (M. Rasmussen and R.

TABLE 6. Effect of potential protease inhibitors on proteolytic activity of *B. fibrisolvens* 49[a]

Compound	Concn (mM)	Mean proteolytic activity[b] ± SD (%) in:	
		Culture fluid	Cells
EDTA	1.0	94 ± 0	92 ± 0
	5.0	92 ± 1	95 ± 6
o-Phenanthroline	0.5	105 ± 3	98 ± 10
	2.5	83 ± 4	106 ± 5
Pepstatin	1.0	85 ± 3	107[c]
p-Chloromercuribenzoate	0.5	102 ± 4	89 ± 7
	2.5	68 ± 6	69 ± 1
Iodoacetamide	1.0	96 ± 1	97 ± 1
	5.0	102 ± 9	96 ± 1
Sodium sulfide	1.0	80 ± 8	85 ± 1
	5.0	95 ± 11	52 ± 2
L-Cysteine	1.0	76 ± 3	76 ± 6
	5.0	65 ± 4	64 ± 10
Dithiothreitol	1.0	68 ± 5	76 ± 11
	5.0	52 ± 7	56 ± 8
TPCK	1.0	97 ± 6	111 ± 16
	5.0	86 ± 6	108 ± 12
TLCK	1.0	82 ± 16	105 ± 8
	5.0	100 ± 23	115 ± 1
PMSF	0.5	11 ± 9	20 ± 16
	1.0	8 ± 5	16 ± 7
Trypsin inhibitor I-S	100[d]	99 ± 1	103 ± 6
	500[d]	97 ± 3	98 ± 13

[a] Cultures were grown on RF medium and were harvested at late-logarithmic-growth phase.
[b] Percent of control activity (no inhibitor added) for which 100% equals 80 and 72 U/ml (culture fluid) or 11 and 4 U/ml (cells). Data are from duplicate samples from two replicate experiments.
[c] Single experiment only.
[d] Concentration is μg/ml.

Hespell, unpublished data). Thus, a portion of the particulate-associated proteolytic activity may be attributed to that found with *B. fibrisolvens* cells per se, as well as the extracellular protease that may be immeshed within the polysaccharide matrix that often surrounds these attached cells (1, 19).

The proteolytic activity of *B. fibrisolvens* is produced constitutively, but the activity level is subject to modulation by growth conditions. With RS medium (Table 1), the growth rate of the organism can be varied over threefold by the use of different carbohydrate growth substrates (Table 4). Under these conditions, proteolytic activity varied twofold and could be positively correlated with growth rate changes. With glucose as the growth substrate, changes in the types and levels of nitrogen sources in the medium resulted in large changes in the relative (units per milligram of protein) proteolytic activity levels (Table 4). These data suggest that exogenous amino acids or peptides may also influence proteolytic activities. In comparison, the proteolytic activity of *Bacteroides amylophilus* is not induced or repressed by the presence of a variety of nitrogen sources or other nutrients in the medium, but the production of proteolytic activity does vary with the growth rate (5, 6).

The proteolytic activity of *B. fibrisolvens* is active over a wide temperature and pH range, with the optimum for the latter between 5.5 and 7.0 (Fig. 1). This is close to the optimal pH range of 6 to 7 that has been reported for mixed ruminal proteases (7, 30). Also, the pH of rumen contents is usually between 5.0 and 7.0. These pH ranges are rather wide for serine proteases which have slightly alkaline optimal pH (4). Since the optimal pH for activity for a protease can be influenced by the substrate in the assay (36, 42), the proteolytic activity of *B. fibrisolvens* may have a different optimal pH when more natural substrates such as ribulose-bis-phosphate carboxylase (a major plant protein) are used. The proteolytic activity was not particularly heat stable, with rapid losses occurring above 47°C, but the protease could be stored at −30°C for 5 to 8 weeks with less than a 10 to 20% loss of activity (data not shown). About 70% of the maximal activity was observed at 39°C, which is the average temperature of the rumen in most animals.

Based on the effects of protease inhibitors, *B. fibrisolvens* produces proteolytic activity characteristic of a serine protease. Treatment with PMSF caused almost a complete inhibition of activity, whereas other serine protease inhibitors (TLCK, TPCK, and trypsin inhibitor I-S) were ineffective at blocking protease activity (Table 6). Because these latter compounds were ineffective, the data suggest that the *B. fibrisolvens* proteolytic activity lacks trypsin- or chymotrypsinlike specificity. The activity may be similar to that of the *Bacillus subtilis* extracellular subtilisin proteases, which are serine proteases and are not inhibited by TLCK or TPCK (35). The lack of inhibition of *B. fibrisolvens* proteolytic activity by EDTA or *o*-phenanthroline suggests the absence of metalloproteases and indicates that the inhibitory effects of dithiothreitol and L-cysteine are not related to the metal chelation properties of these sulfhydryl compounds. These inhibitory effects, as well as the lack of inhibition by both *p*-chloromercuribenzoate and iodoacetamide, are contrary to what one expects if sulfhydryl proteases are present.

Both the cell-associated and culture fluid activities had similar properties with respect to temperature stability, optimal pH (Fig. 1), and sensitivity to protease inhibitors (Table 6). Taken collectively, these data strongly suggest that the two activities are catalyzed by the same or similar enzyme(s). Attempts to purify and characterize the cell-associated activity in more detail were not undertaken at this time because it represented only about 5% of the total culture activity.

Attempts were made to purify the extracellular proteolytic activity for biochemical characterization. These attempts were generally unsatisfactory, as low yields were obtained and little purification was achieved. Preliminary data from SDS-PAGE indicate that the proteolytic activity may be catalyzed by a 30-kDa protein. Serine proteases are generally in the range of 20 to 40 kDa. However, *Streptomyces fradiae* and a *Sorangium* species (36) produce extracellular proteases of 14 and 19 kDa, respectively. The most surprising outcome of our initial purification attempts was the association of the proteolytic activity with a carbohydrate material. This material appears to be a high-molecular-weight polysaccharide, because it can be sedimented along with proteolytic activity from culture fluids. The nature of the association between the proteolytic enzyme(s) and the polysaccharide is unknown. This polysaccharide association has precluded us from purifying the protease by conventional procedures at this time. Studies are in progress to characterize this polysaccharide and to compare its composition with that of the extracellular polysaccharide slimes made by various *B. fibrisolvens* strains and other ruminal bacterial species, such as *Ruminococcus albus* (41).

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